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Cryptosporidium ryanae n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos taurus*)

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Abstract

A new species, *Cryptosporidium ryanae*, is described from cattle. Oocysts of *C. ryanae*, previously identified as the *Cryptosporidium* deer-like genotype and recorded as such in GenBank (AY587166, EU203216, DQ182597, AY741309, and DQ871345), are similar to those of *Cryptosporidium parvum* and *Cryptosporidium bovis* but smaller. This genotype has been reported to be prevalent in cattle worldwide. Oocysts obtained from a calf for the present study are the smallest *Cryptosporidium* oocysts reported in mammals, measuring 2.94–4.41 μm × 2.94–3.68 μm (mean = 3.16 μm × 3.73 μm) with a length/width shape index of 1.18 ($n = 40$). The pre-patent period for two *Cryptosporidium*-naïve calves fed *C. ryanae* oocysts was 11 days and the patent period was 15–17 days. Oocysts were not infectious for BALB/c mice or lambs. Fragments of the SSU-rDNA, HSP-70, and actin genes amplified by PCR were purified and PCR products were sequenced. Multi-locus analysis of the three unlinked loci demonstrated the new species to be distinct from all other species and also demonstrated a lack of recombination, providing further evidence of species status. Based on morphological, molecular and biological data, this geographically widespread parasite found only in *Bos taurus* calves is recognized as a new species and is named *C. ryanae*.
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Keywords: Cryptosporidiosis; New species; Cattle; Taxonomy; Molecular

1. Introduction

Cattle have been reported as primary hosts for three species and one genotype of *Cryptosporidium*: *C. parvum*, *C. andersoni*, *C. bovis*, and the *Cryptosporidium* deer-like genotype (Lindsay et al., 2000; Santín et al., 2004; Fayer et al., 2005, 2006, 2007; Feng et al., 2007). Although the *Cryptosporidium* deer-like genotype has never been found in deer it was identified as ''deer-like'' because its SSU-rDNA gene sequence was very similar to that of the *Cryptosporidium* deer genotype (AY120910) reported by Xiao et al. (2002). Nucleotide sequence fragments of the SSU-rDNA gene have been deposited in Genbank as AY587166 (Santín et al., 2004), DQ871345 (Feng et al., 2007), and EU203216 (Feltus et al., 2008); nucleotide sequence fragments of the actin and HSP-70 gene have been deposited in Genbank as AY741309 (Fayer et al., 2005) and DQ182597 (Langkjær et al., 2007), respectively. In earlier studies of calves on 14 dairy farms in 7 states 3.8% of 1411 dairy cattle 5 days to 2 years of age were found infected with the *Cryptosporidium* deer-like genotype (Santín et al., 2004; Fayer et al., 2006). This genotype also was found in 18 of 30 calves that were examined repeatedly over a period of 2 years on a dairy farm in Maryland (Santín et al., 2008).

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Genetically based studies have detected the *Cryptosporidium* deer-like genotype in cattle in China, Denmark, Hungary, Kenya, Malaysia, Northern Ireland, Zambia, the UK, and the USA (Santín et al., 2004; Fayer et al., 2006, 2007; Feng et al., 2007; Langkjær et al., 2007; Plutzer and Karanis, 2007; Siwila et al., 2007; Thompson et al., 2007; Brook et al., 2008; Feltus et al., 2008; Halim et al., 2008; Szonyi et al., 2008).

Differences related to the age of infected cattle have been observed in the prevalence of species and genotypes of *Cryptosporidium*. Eight of 393 pre-weaned calves (5 days to 2 months of age) and 36 of 474 post-weaned calves (3–11 months of age) were found infected with the *Cryptosporidium* deer-like genotype (Santín et al., 2004). On most of the same farms 10 of 571 heifers (1–2-year of age) were also found infected with the *Cryptosporidium* deer-like genotype (Fayer et al., 2006). The deer-like genotype was identified in one post-weaned and five pre-weaned calves in Georgia, USA, one pre-weaned calf in China (Feng et al., 2007), two 14-day-old calves in Kenya (Szonyi et al., 2008), and three 1–6-months-old calves in Malaysia (Halim et al., 2008). In addition, three pre-weaned calves and a milking cow in Georgia had concurrent infections with the deer-like genotype and *C. bovis* (Feng et al., 2007). Of 1150 cattle from 50 dairy herds in Denmark, 154 specimens were *Cryptosporidium*-positive and 14% of these were positive for the deer-like genotype, all in older calves (Langkjær et al., 2007). In calves (median age 26 days) from 41 farms in the UK, 60 specimens were *Cryptosporidium*-positive and only one of those was the deer-like genotype (Brook et al., 2008).

Based on the unique molecular characteristics of the deer-like genotype and its presence only in cattle, the present study was conducted to determine if additional unique attributes were present to identify it as a separate species. As proposed by Xiao et al. (2004) for qualification of species status, morphometric studies were conducted, biological data were obtained, and three unlinked loci were sequenced and compared with sequences for the same genes from other species and genotypes of *Cryptosporidium*. Data obtained in the present study indicate that the *Cryptosporidium* deer-like genotype qualifies as a new species and as such is named *Cryptosporidium ryanae* in honor of Dr. Una Ryan.

2. Materials and methods

2.1. Collection of parasites

Feces were collected directly from the rectum of 18–20-week-old dairy calves on a dairy farm in Maryland to obtain *C. ryanae* oocysts for the present study. Feces were collected from a group of 29 calves and later from a different group of 25 calves on the same farm.

Oocysts of *C. ryanae* from the feces of an 18-week-old calf (calf no. 1) at the Maryland dairy farm were used to obtain oocyst measurements and to experimentally infect two *Cryptosporidium*-naïve calves (calf nos. 2 and 3). Feces from calves 2 and 3 were collected daily and placed in individual plastic specimen cups and held at 5 °C until processed for examination to determine the pre-patent and patent periods. Oocysts of *C. ryanae* used to determine infectivity for additional animal species were obtained from the feces of two 20-week-old calves (calf nos. 4 and 5) at the dairy farm and were used to determine infectivity of this species for mice and lambs.

To obtain oocysts for microscopic examination and for DNA extraction, feces were processed as described (Fayer et al., 2000). Fifteen grams of feces were transferred from each specimen cup to a 50-ml centrifuge tube containing approximately 35 ml dH_2O. The tube was capped and contents were thoroughly mixed using a Vortex-Genie (Scientific Industries, Bohemia, New York). To remove large particulates, the fecal suspension was poured through a 45-μm pore size wire screen into another 50-ml tube. The final volume was adjusted to 50 ml with dH_2O and the tube was centrifuged at $1800 \times g$ for 15 min. Supernatant was discarded, and the pellet, suspended in 25 ml dH_2O, was mixed by Vortex-Genie. Twenty-five milliliters of CsCl (1.4 g/l) was added to the tube, the contents were mixed thoroughly, and the tube was centrifuged at $300 \times g$ for 20 min. Four milliliters of supernatant were aspirated from the top of the tube and transferred to a 15-ml centrifuge tube where dH_2O was added to reach a final volume of 15 ml. The tube was centrifuged at $1800 \times g$ for 15 min and similarly washed twice with dH_2O before the final pellet was suspended in 500 μl of dH_2O. Portions of the suspension were examined by immunofluorescence microscopy (IFA), differential interference microscopy, and molecular methods as described below.

2.2. Microscopic examination of oocysts

A 100-μl aliquot of cleaned oocyst suspension transferred to a microcentrifuge tube was centrifuged and the pellet was suspended in 50 μl of pre-mixed anti-*Cryptosporidium* reagent (Mer*IFluor*™, Meridian Biosciences Inc., Cincinnati, Ohio). Two microliters of suspension was pipetted into each well (11-mm diameter) of a 3-well glass microscope slide and the

slide covered with a coverslip. The entire well area was examined by fluorescence microscopy at 400× using a Zeiss Axioskop equipped with epifluorescence and an FITC-Texas Red™ dual wavelength filter.

After detection by IFA, oocysts of *C. ryanae* from naturally infected calf no. 1 were suspended in water, placed on a glass microscope slide with a coverslip, and 40 oocysts measured by ocular micrometer at 1000× using a Zeiss Axioskop microscope with differential interference contrast microscopy. A photomicrograph of *C. ryanae* oocysts observed by differential interference contrast microscopy was deposited as a phototype in the U.S. National Parasite Collection, Beltsville, Maryland. For purposes of comparison, oocysts ($n = 40$) of *C. parvum* from another bovine source were measured by the same person using the same microscope.

2.3. DNA extraction, PCR, and sequence analyses

Total DNA was extracted from each 50 μl suspension of cleaned oocysts using a DNeasy Tissue Kit (Qiagen, Valencia, California). To increase the quantity of recovered DNA the nucleic acid was eluted in 100 μl of elution buffer included in the DNeasy Kit.

Fragments of SSU-rDNA (~830 bp), HSP-70 (~325 bp), and actin (~1066 bp) genes were amplified by PCR (Xiao et al., 1999; Morgan et al., 2001; Sulaiman et al., 2002). PCR products analyzed on 1% agarose gel were visualized by ethidium bromide staining and purified with Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT™) (USB Corporation, Cleveland, Ohio). Purified products were sequenced in both directions with the same PCR primers in 10 μl reactions, Big Dye™ chemistries, and an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, California).

2.4. Phylogenetic analyses

The 18S rDNA, HSP-70, and actin sequences were then compared with sequences obtained from other *Cryptosporidium* species and genotypes from GenBank. *Plasmodium falciparum* was used as an out-group for HSP-70 (GenBank accession no. M19753), actin (GenBank accession no. M19146) and SSU-rDNA (GenBank accession no. M19172) analyses as in previous studies (Ryan et al., 2003, 2004; Fayer et al., 2005; Ng et al., 2006). Sequences were aligned following the Clustal W algorithm included in the Megalign module (DNASTAR Inc., Madison, Wisconsin). The use of Clustal W determines that once a gap is inserted it can be removed only by editing. Therefore, final alignment adjustments were made manually to remove artificial gaps. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar et al., 2004). Phylogenetic inference was performed by the Neighbor-Joining (NJ) method of Saitou and Nei (1987).

Nucleotide sequences of the SSU-rDNA, HSP-70, and actin genes of *C. ryanae* have been deposited in GenBank under the accession numbers EU410344–EU410346, respectively.

2.5. Infectivity for calves

Oocysts from naturally infected calf no. 1, identified as *C. ryanae* by PCR and SSU-rDNA gene sequencing, were fed to two colostrum deprived Holstein-Friesian male calves (calves no. 2 and 3). To prevent accidental infection with *Cryptosporidium* species in the environment calves were taken from their dams at 1 day of age and housed separately on wood shavings in 3 m^2 pens with cement walls and floor in an isolated cinderblock building. Animal caretakers wore new disposal coveralls, shoe covers and gloves whenever they entered the area where calves were housed. Calves were fed calf milk-replacer twice daily and water was available *ad libitum*. Calves no. 2 and 3 were 17 and 18 days of age, respectively, when fed an unknown number of *C. ryanae* oocysts from calf no. 1 suspended in water. Feces were then collected from each calf daily for 30 consecutive days beginning the day after oocysts were fed.

2.6. Infectivity for lambs

Three colostrum-deprived neonatal Suffolk lambs were purchased from a local farmer. They were housed and cared for in a similar building and with the same precautions to prevent accidental infection as for the calves. They were fed lamb milk-replacer two to three times a day via a nippled bottle. Water was available *ad libitum*. Oocysts, identified as *C. ryanae* by PCR and SSU-rDNA gene sequencing were obtained from naturally infected calves no. 4 and 5, The lambs, at 13-, 17-, and 18-days of age, respectively, were each fed 4000 oocysts suspended in milk via a nippled bottle. Feces were then collected from each lamb for 21 consecutive days beginning the day after oocysts were fed.

2.7. Infectivity for mice

Two litters of 5-day-old BALB/c mice with their dams were purchased from the National Cancer Institute (Frederick, Maryland). Five hundred oocysts of *C. ryanae*

were administered to each of three mouse pups at 5 days of age and to three additional mouse pups at 14 days of age by gastric intubation using a 26-ga gavage needle fitted to a micropipette. Six other mice received oocysts of *C. parvum* (Beltsville strain) obtained from experimentally infected calves and served as positive controls. Mice were killed by CO_2 asphyxiation followed by cervical dislocation 5 days after intubation. Tissues from stomach, duodenum, jejunum, and ileum were obtained from each mouse and subjected to DNA extraction and PCR (as described above).

2.8. Animal care

All housing, feeding, and experimental procedures involving calves, lambs, and mice were conducted under protocols approved by the Beltsville Area Animal Care and Use Committee.

3. Results

3.1. Prevalence of C. ryanae

Of the first group of twenty-nine 18–20-week-old dairy calves, oocysts of *C. ryanae* were detected in the feces of five calves (17.2%). Four of the five calves excreted very few oocysts per g of feces. Calf no. 1 excreted the most oocysts and these were measured, photographed, and used to infect calves no. 2 and 3. A second group of 20 dairy calves was examined and *C. ryanae* was detected in two calves (10%). Oocysts from the two calves were pooled and used to infect lambs and mice.

3.2. Oocyst characteristics

Oocysts of *C. ryanae* stained with anti-*Cryptosporidium* reagent and examined by fluorescence microscopy appeared pale in comparison with those of *C. parvum* which appeared bright green. Oocysts ($n = 40$) of *C. ryanae* measured 2.92–4.41 μm × 2.94–3.68 μm with a mean size of 3.73 μm × 3.16 μm and a shape index of 1.18. Oocysts ($n = 40$) of *C. parvum* measured 4.41–5.88 μm × 4.41–5.88 μm with a mean size of 5.41 μm × 4.93 μm and a length/width shape index of 1.10. One or two sporozoites per oocyst of *C. ryanae* could be seen in different focal planes but the total number in each oocyst could not be clearly visualized.

3.3. Gene sequence data

Partial sequences of the SSU-rDNA, actin, and HSP-70 genes were compared with *Cryptosporidium* sequence data obtained from GenBank. Phylogenetic relationships for these three genes were consistent, with *C. ryanae* forming a distinct cluster with the *C. bovis* and the deer genotype (Figs. 1–3).

3.4. Transmission studies

Calves no. 2 and 3 began excreting *C. ryanae* oocysts 11 days after they were fed a suspension of oocysts from calf no. 1. Oocysts were detected in feces for 15 and 17 consecutive days, respectively, for calves 3 and 2 by PCR and microscopy. Genetic analysis of all these samples revealed that both calves were excreting *C. ryanae* oocysts.

Of the three lambs fed *C. ryanae* oocysts none excreted detectable oocysts over the following 21 days. Of the six mice intubated with oocysts of *C. ryanae*, DNA of *C. ryanae* was not detected in their gastrointestinal tissues whereas DNA of *C. parvum* was detected in ileal tissue of all positive control mice.

3.5. Description

3.5.1. Cryptosporidium ryanae n. sp.

Diagnosis	Oocysts are shed in feces fully sporulated. Oocysts ($n = 40$) measure 2.94–4.41 μm × 2.94–3.68 μm with a mean size of 3.73 μm × 3.16 μm and a length/width shape index of 1.18. These are the smallest oocysts reported for a *Cryptosporidium* sp. infecting a mammalian host. Endogenous stages are unknown
Type host	Cattle, *Bos taurus*
Other natural hosts	None known
Experimental transmission	Attempts to infect cattle were successful. Attempts to infect BALB/c mice and lambs were unsuccessful
Pre-patent period	11 days
Patent period	15–17 days
Type locality	Maryland, USA
Other localities	Denmark, Hungary, China, Kenya, Malaysia, Northern Ireland, the United Kingdom, and Zambia
Material deposited	A phototype and description of oocysts is deposited in the United States National Parasite Collection, Beltsville, Maryland as USNPC 100508
Etymology	This species is named *Cryptosporidium ryanae* in honor of Dr. Una Ryan who has contributed greatly to the taxonomy of *Cryptosporidium* species

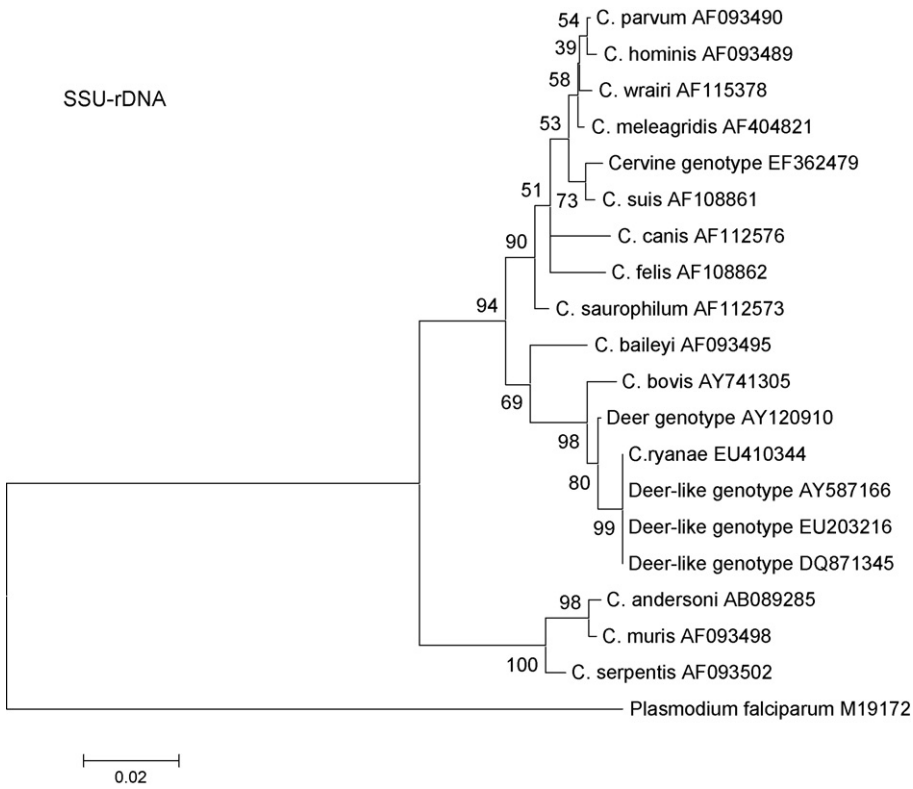

Fig. 1. Phylogenetic relationships among *Cryptosporidium* species and genotypes inferred by a neighbor-joining analysis of a fragment of the 18S rRNA gene sequence, based on genetic distances calculated by the Kimura two-parameter model. Numbers on branches are percent bootstrapping values from 1000 replicates.

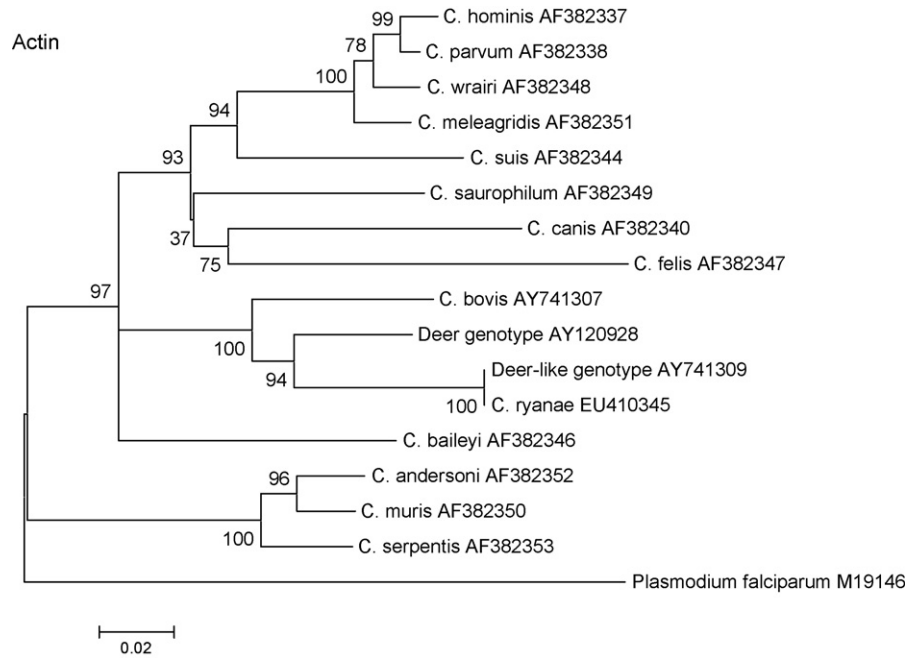

Fig. 2. Phylogenetic relationships among *Cryptosporidium* species and genotypes inferred by a neighbor-joining analysis of a fragment of the actin gene sequence, based on genetic distances calculated by the Kimura two-parameter model. Numbers on branches are percent bootstrapping values from 1000 replicates.

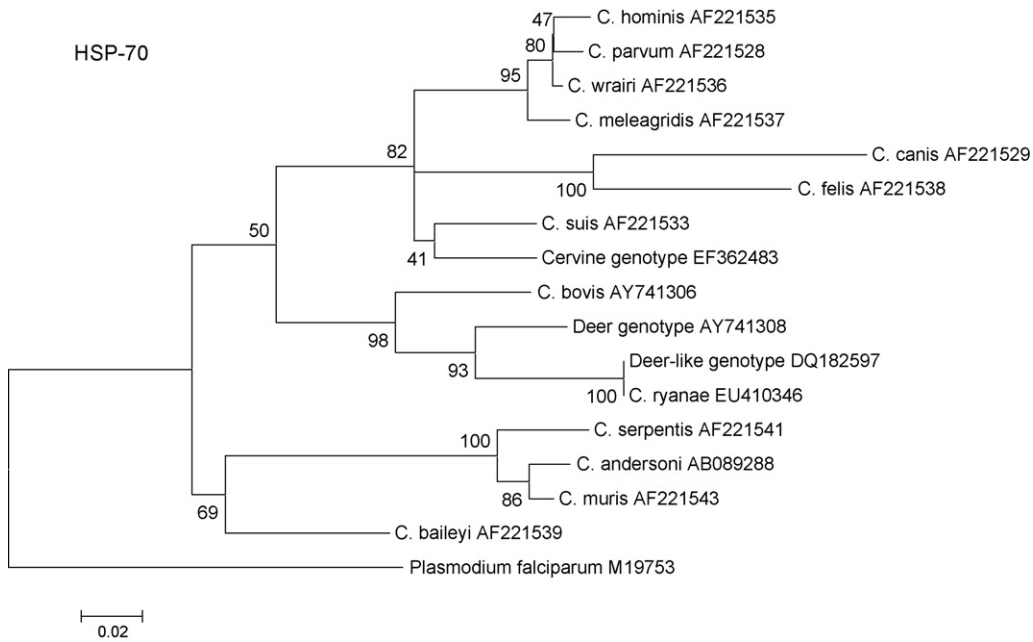

Fig. 3. Phylogenetic relationships among *Cryptosporidium* species and genotypes inferred by a neighbor-joining analysis of a fragment of the HSP-70 gene sequence, based on genetic distances calculated by the Kimura two-parameter model. Numbers on branches are percent bootstrapping values from 1000 replicates.

4. Discussion

Oocysts of *C. ryanae* were found to be morphologically distinguishable from those of *C. parvum*, *C. bovis*, and *C. andersoni*, the other species routinely found to infect cattle. Because of the small size of *C. ryanae* oocysts, all sporozoites could not be clearly seen within the oocysts. Forty oocysts of *C. ryanae* had a mean size of 3.73 μm × 3.16 μm whereas 40 oocysts of *C. parvum* obtained from another calf, processed, and measured under the same conditions by the same person in the present study had a mean size of 5.41 μm × 4.93 μm. Mean sizes reported for *C. parvum* oocysts in other studies were 5.0 μm × 4.5 μm, 5.0 × 4.7 μm, 5.2 × 4.3 μm and 5.47 μm × 5.15 μm (Upton and Current, 1985; Fayer et al., 2001, 2005; Fall et al., 2003), all very close to one another but slightly larger than the mean size of 4.89 μm × 4.63 μm found for oocysts of *C. bovis* (Fayer et al., 2005) which in turn are larger than those of *C. ryanae*. Although smaller than other species of *Cryptosporidium* in cattle, oocysts of *C. ryanae* are only slightly less round, having a length/width shape index of 1.18, whereas oocysts of *C. parvum* and *C. bovis* have a shape index of 1.06 (Fayer et al., 2005) and those of *C. parvum* measured in the present study had a shape index of 1.10. Oocyst characteristics such as color and thickness of the oocyst wall were indistinguishable among the three species.

Genetically confirmed infections with *C. ryanae*, previously referred to as the deer-like genotype, have been found in dairy and beef cattle worldwide. Within the United States cattle infected with *C. ryanae* were geographically widespread, covering an overall distance of over 2100 km, on farms with a wide range of climatic, soil, and management conditions (Santín et al., 2004; Fayer et al., 2006). In those point prevalence studies the percent of infected cattle appeared small with only 2% of 393 pre-weaned calves, 7.6% of 474 post-weaned calves (3–11 months of age) and 1.8% of 571 heifer calves infected with *C. ryanae* (Santín et al., 2004; Fayer et al., 2006). In a longitudinal study of 30 calves from birth to 2 years of age 60% became infected with *C. ryanae* (Santín et al., 2008). In that study, *C. ryanae* was detected in calves from 2.5–16 months of age, with the greatest number of infected calves found at 18–20 weeks of age (Santín et al., 2008). All calves in that study previously had been infected with *C. parvum*. From the same farm where the longitudinal study was conducted, a single fecal sample was collected from 49 calves for the present study and 14.9% of those 18–20-week-old calves were found infected.

A biologic difference between *C. ryanae* and *C. parvum* is an age related bias; 85% versus 5% of *Cryptosporidium*-positive dairy calves 2 months of age and younger were infected with *C. parvum* versus *C. ryanae*, whereas 31% versus 1% of dairy calves 3–11

months of age were infected with *C. ryanae* versus *C. parvum* (Santín et al., 2004). In that study, another distinct biological feature was reported. None of the calves infected with *C. ryanae* had any signs of disease, whereas calves infected with *C. parvum* often have been reported as diarrheic (de Graaf et al., 1999).

Phylogenetic analyses confirmed the validity of *C. ryanae* at three independent loci. Datasets for these three loci provide strong support for the genetic distinctiveness of *C. ryanae* from *C. parvum* and indicate the clustering of *C. ryanae* with *C. bovis* and the *Cryptosporidium* deer genotype (Figs. 1–3). Phylogenetic analyses of the SSU rDNA locus revealed that *C. ryanae* had a sequence similarity of only 93.9% with *C. parvum* and 93.6% with *C. hominis*. Currently recognized species of *Cryptosporidium* share much greater similarities such as *C. meleagridis*, *C. parvum*, and *C. hominis* (98.5–99.1%), and *C. parvum* versus *C. hominis* (99.1%).

Genetic differences from three independent loci combined with biological differences such as strict host specificity for cattle, the age bias for infection of post-weaned cattle under field conditions, a lack of cross-species immunity (as observed in natural infections with *C. ryanae* following infection with *C. parvum*; Santín et al., 2008), and the lack of pathogenicity indicate that *C. ryanae* is a distinct species. The correct identification of *Cryptosporidium* species in clinical and epidemiological specimens has important and far reaching veterinary and public health implications. Cattle of all ages have been found infected with *Cryptosporidium*. Some animals suffer severe illness and others appear healthy. The diagnosis of infection, often made by microscopic identification of the oocyst stage, but infrequently confirmed by molecular methods, has led to the widespread misconception that cattle of all ages are major sources of the zoonotic species *C. parvum* that is pathogenic for humans, cattle, and other animals. Identification of *C. bovis* and *C. ryanae* as the primary species of *Cryptosporidium* found in post-weaned cattle, species neither found to infect humans nor to cause illness in livestock, provides clarification to the complicated epidemiologic paradigm associated with the genus *Cryptosporidium*. Establishment of the species *C. ryanae* from the former deer-like genotype should also clarify the confusion that exists and the eliminate future errors in publications in reference to the other genotypes now identified as the deer genotype and the cervine genotype.

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